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THE NORADRENALINE REUPTAKE INHIBITOR ATOMOXETINE PHASE-SHIFTS THE CIRCADIAN CLOCK IN MICE

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Abstract—Circadian rhythms are recurring cycles in physiology and behaviour that repeat with periods of near 24 h and are driven by an endogenous circadian timekeeping system with a master circadian pacemaker located in the suprachiasmatic nucleus (SCN). Atomoxetine is a specific noradrenaline reuptake inhibitor that is used in the clinical management of attention-deficit/hyperactivity disorder (ADHD). In the current study we examined the effects of atomoxetine on circadian rhythms in mice. Atomoxetine (i.p.; 3 mg/kg) treatment of mice free-running in constant light (LL) at circadian time (CT) 6 induced large phase delays that were significantly different to saline controls. Treatment of animals with atomoxetine at CT13 or CT18 did not elicit any significant phase shifts. We also examined the effects of atomoxetine treatment of animals free-running in constant darkness (DD). Atomoxetine treatment at CT6 in these animals leads to more modest, but significant, phase advances, whereas treatment at CT18 did not elicit significant phase shifts. The effects of atomoxetine in LL were attenuated by pretreatment with the α-1 adrenoreceptor antagonist prazosin and were mimicked by another noradrenaline reuptake inhibitor, reboxetine. Further, atomoxetine treatment at CT6 induced a downregulation of c-Fos and CLOCK in the SCN, but did not alter the expression of PER2 and BMAL1. Atomoxetine during the night phase did not alter any of these factors. Atomoxetine treatment preceding a light pulse at CT15 enhanced the magnitude of the photic-phase shift, whereas it altered photic induction of the immediate early gene products c-Fos and ARC in the SCN. These data indicate that atomoxetine can reset the circadian clock and indicate that part of the therapeutic profile of atomoxetine may be through circadian rhythm modulation.

Key words: atomoxetine, circadian, suprachiasmatic, reboxetine, c-Fos, clock.

Circadian rhythms are near 24-h recurring cycles in a host of behavioural and physiological parameters that are underpinned by an endogenous circadian timekeeping system (Reppert and Weaver, 2002). In mammals the master pacemaker of this circadian system is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which receives afferent input allowing the entrainment of the circadian clock to appropriate sensory and environmental time cues (zeitgebers), the dominant one being light (Dibner et al., 2010). The molecular machinery that allows for stable generation of such circadian rhythms is now known to consist of a panel of clock genes that regulate both their own expression and also those of clock-controlled genes, with various studies demonstrating that approximately 10% of the transcriptome of various tissues displays significant circadian rhythmicity (Hogenesch et al., 2003).

The SCN is known to receive input from a number of midbrain and brainstem regions (Moga and Moore, 1997) as well as projecting indirectly to key brainstem areas such as the locus coereleus in order to drive circadian rhythms in arousal (Aston-Jones et al., 2001). Some input pathways have received sustained attention in the research literature (e.g. the serotoninergic input; Morin and Allen, 2006), whereas other systems have received somewhat less attention. The SCN is known to receive noradrenergic innervation (Cagampang et al., 1994; Jacomy and Bosler, 1995), with noradrenaline (NA) levels displaying rhythms in the rat and hamster SCN (Semba et al., 1984; Cagampang et al., 1994) as well as throughout the rest of the brain (e.g. Manshardt and Wurtman, 1968). The role of NA in regulating SCN function is not clear, although it has been proposed that NA regulates the expression of the key SCN neuropeptides arginine vasopressin and vasoactive intestinal polypeptide (Vacher et al., 2003, 2004). Further, it has been noted that the SCN expresses both α-1 and α-2 adrenoreceptors (Morien et al., 1999). There have been a limited number of studies examining the effects of adrenergic agents on circadian organization of behaviour and clock gene regulation (Rosenwasser, 1996; Terazono et al., 2003; Wongchitrat et al., 2009) with many issues regarding the adrenergic regulation of the master SCN circadian clock remaining unascertained. One such issue is the extent to which medication used to treat affective and other psychiatric disorders, which target the noradrenergic system, may exert their effects through a circadian modulation. For example, the noradrenaline reuptake inhibitor reboxetine is licensed for use in the treatment of major depression, which itself has long been associated with circadian desynchrony (Wirz-Justice, 2009). Further, another noradrenaline reuptake inhibitor, atomoxetine, is licensed for the treatment of attention deficit/hyperactivity disorder (ADHD), which again has been associated with phase misalignment of the circadian clock (Van Veen et al., 2010). Given that correction of these circadian discrepancies is associated with symptom relief (Coogan and

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Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CT, circadian time; DD, constant darkness; IEG, immediate early gene; LL, constant light; NA, noradrenaline; SCN, suprachiasmatic nucleus.

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Thome, 2011), it is of interest to inquire as to the actions of noradrenergic modulating drugs on circadian rhythms. To this end we have studied the effects of atomoxetine on circadian rhythms in the mouse in order to gain insight into putative mechanisms that may be of importance in human psychopharmacology. As non-photic stimuli that phase-shift the clock also suppress expression of clock genes in the SCN (e.g. Maywood et al., 1999) we have also examined the effect of atomoxetine on the expression of a number of clock gene protein products.

EXPERIMENTAL PROCEDURES

Animals and housing

Male C57BL/6 mice (22–25 g) obtained from Harlan Laboratories (Leicestershire, UK) were used throughout this study. Animals were singly housed in polypropylene cages equipped with running wheels (11 cm diameter) with food and water available *ad libitum* and temperature held constant at 21±1 °C and humidity at 50±10%. Cages were then housed in an environmental isolation cabinet to allow for full control of the photic environment. They were illuminated using a fluorescent white light source, with average illuminance of 250 lx at the level of the cage floor when lights were on. Bedding was changed every 14 days, and never in the period leading up to or after a pharmacological intervention. All experiments were approved by the Research Ethics Committee, National University of Ireland Maynooth and licensed by the Irish Department of Health and Children and conformed to the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to reduce the number of animals used in the study, as well as any pain and suffering.

Circadian rhythm monitoring

Wheel running was monitored through microswitches attached to the running wheels communicating with the Chronobiology Kit (Stanford Software Systems, CA, USA) to allow for production of actograms of wheel-running behaviour. Before any pharmacological intervention animals were allowed to free-run for 2 weeks to allow for analysis of stable baselines of circadian factors. Phase shifts were rated by two to three independent observers blind to the treatments by means of the line-of-best-fit method through activity onsets for 7 days before and after any intervention. Phase shifts were calculated as the difference in the regression lines before and after the intervention, calculated on the day following the intervention. Period length and rhythm strength were obtained from Poisson Periodograms in the Chronobiology kit.

Drugs

Atomoxetine HCl was obtained from Tocris Bioscience (Bristol, UK), dissolved in 0.9% sterile saline and administered i.p. at a dose of 3 mg/kg, a dose which has been previously shown to elicit neurochemical effects in rodent brain (e.g. Bymaster et al., 2002). Prazosin and reboxetine were also obtained from Tocris Bioscience and administered at doses of 4 mg/kg i.p. and 20 mg/kg i.p., doses previously determined to be effective in similar approaches (Cryan et al., 2004).

Effects of atomoxetine and reboxetine on free running circadian rhythms

In these experiments, animals were maintained in cages with running wheels under a 12:12 light:dark cycle for 2 weeks before being released into constant conditions (either constant light (LL) or constant darkness (DD)). Both photic backgrounds were used, as previous research has indicated that LL enhances phase-shifting in response to some stimuli (Knoch et al., 2004). Animals were allowed to free run for 2 weeks before the first drug treatment. Animals (n=8 for LL and n=8 for DD) received an injection of atomoxetine (3 mg/kg) and an injection of saline at circadian time (CT) 6, each treatment separated by 14 days before being crossed over to the other treatment, such that each animal received both atomoxetine and vehicle. Further to this, each animal then received atomoxetine or saline at CT18.

To further test the phase-specificity of atomoxetine's actions a separate group of mice (n=8) were free-run in LL and in a counterbalanced cross-over design received atomoxetine/saline at CT13 followed by the complementary treatment 2 weeks later, followed by a further 14-day free running to allow for accurate rating of the stable phase shift. Timepoints were selected on the basis of previous studies of phase-dependence of non-photic and pharmacological manipulations that produce phase shifts, which typically indicated efficacy when applied in the subjective day but not the subjective night.

To test whether another noradrenaline reuptake inhibitor, reboxetine, would have a similar effect as to atomoxetine, mice (n=8) were free-run in LL for 2 weeks before receiving either reboxetine (20 mg/kg, i.p.) or saline, before receiving the complementary treatment 14 days later, followed by a further 14 days of free running. To test whether the effects of atomoxetine were modulated by an adrenergic receptor antagonist, prazosin, a group of mice (n=8) were free-run in LL for 14 days before receiving treatment with atomoxetine at CT6 coupled with either prazosin treatment (4 mg/kg, i.p.; n=4) or saline treatment (n=4) 30 min before the atomoxetine treatment (so at CT5.5). After a further 14 days animals again received atomoxetine at CT6 preceded by either saline or prazosin in a cross-over design, so that each animal received atomoxetine plus prazosin and atomoxetine plus saline.

Effects of atomoxetine in modulating photic phase shifts

In this experiment we examined whether atomoxetine pretreatment would alter light-induced phase shifts of the circadian rhythm. Mice (n=12) were free-run in DD for 2 weeks, before receiving a 30 min light pulse (150 lx) at CT15-CT15.5, the circadian phase at which a maximal phase delay is elicited in mice (e.g. Pendergast et al., 2010). Thirty min before the light pulse animals received an i.p. injection of either atomoxetine (3 mg/kg) or saline. Animals were then allowed to free run for another 14 days before receiving another light pulse at CT15 crossed over to the complementary atomoxetine/saline treatment, so that each animal received two light pulses at CT15, one preceded with atomoxetine and one with saline.

Effects of atomoxetine on clock gene production expression in the SCN

At the end of each behavioural experiment animals received a final treatment and then were killed by cervical dislocation and their brains removed and fixed for immunohistochemical processing. Animals received atomoxetine or saline at CT6 in LL, atomoxetine or saline at CT13 in LL, atomoxetine or saline at CT6 in DD, reboxetine or saline in LL at CT6 and atomoxetine at CT6 preceded by saline at CT5.5 or atomoxetine at CT6 preceded by prazosin at CT5.5 (n=4 for each treatment group). Animals were sampled and brains removed 2 h following the final treatment. For light pulse experiments animals received saline plus a 30-min light pulse at CT15 (n=4) or atomoxetine at CT14.5 followed by a 30-min light pulse at CT15 (n=4) before being sampled 1 h after the onset of the light pulse. Brains were immersion fixed as previously described (Beynon et al., 2009) before being cryoprotected in 30% sucrose. Brains were then sectioned coronally into